RESEARCH COMMUNICATION

The adrenal capsule is a signaling center controlling cell renewal and zonation through *Rspo3*

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Adrenal glands are zonated endocrine organs that are essential in controlling body homeostasis. How zonation is induced and maintained and how renewal of the adrenal cortex is ensured remain a mystery. Here we show that capsular RSPO3 signals to the underlying steroidogenic compartment to induce β-catenin signaling and imprint glomerulosa cell fate. Deletion of RSPO3 leads to loss of SHH signaling and impaired organ growth. Importantly, *Rspo3* function remains essential in adult life to ensure replenishment of lost cells and maintain the properties of the zona glomerulosa. Thus, the adrenal capsule acts as a central signaling center that ensures replacement of damaged cells and is required to maintain zonation throughout life.

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The mammalian adrenal gland is derived from the adrenal primordium, a precursor of the adrenal cortex that becomes invaded by neural crest cells, which will later form the adrenal medulla. By embryonic day 12.5 (E12.5) in mice, the developing organ becomes surrounded by condensing mesenchymal cells that ultimately form the capsule (Bandiera et al. 2013). A second contribution to the capsule is likely derived from adrenal progenitor cells that have lost SF1 expression (Wood et al. 2013). The permanent cortex is formed through recruitment of capsular cells in a process that involves SHH signaling (King et al. 2009). By E17.5, steroidogenic cells have adopted specific expression profiles, with the outermost cell layers (zona glomerulosa [ZG]) producing enzymes that are required for mineralocorticoid production (e.g., CYP11B2), and deeper layers (zona fasciculata [ZF]) expressing genes involved in glucocorticoid synthesis (*Cyp11b1*). In humans, but not rodents, a third layer (zona reticularis) can be distinguished that produces androgens and is located close to the medulla. Several lines of evidence suggest that β-catenin plays an important role in adrenal zonation and maintenance. Activation of the β-catenin pathway is restricted to the ZG (Kim et al. 2008; Walczak et al. 2014), and ectopic expression leads to the activation of ZG markers in ZF cells (Berthon et al. 2010). Moreover, β-catenin seems to bind to and control the expression of *At1r*, a gene specifically expressed within the ZG (Berthon et al. 2014). Finally, stochastic deletion of β-catenin using a low-level expressing *Sf1-Cre* line leads to progressive loss of the adrenal cortex in adult life (Kim et al. 2008).

Results and Discussion

Recent lineage tracing studies have revealed that adrenal maintenance likely involves cell conversion of ZG cells into ZF cells (Freedman et al. 2013), with cells being displaced in a centripetal fashion. In our search for signaling molecules that may be involved in adrenal cell renewal and zonation, we identified members of the *R-spondin* gene family to be expressed from E12.5 onward within mesenchymal cells surrounding the forming adrenal (Supplemental Fig. S1). Expression of *Rspo1* and *Rspo3* was maintained throughout development and well into adulthood (Fig. 1A; Supplemental Fig. S1A). R-spondins are signaling molecules that bind to LGR receptors and positively modulate the β-catenin signaling pathway (de Lau et al. 2014). As expected from previous studies, β-catenin was highly expressed in the ZG (Fig. 1B). Moreover, immunofluorescence analysis revealed LGR5 to be enriched within ZG cells (Fig. 1B), which is consistent with an active RSPO/LGR5/β-catenin cascade.

The adrenal capsule consists of mesenchymal cells that are positive for the SHH mediator GLI1 (King et al. 2009) and COUPTFII (also known as NR2F2) (Wood et al. 2013) and surrounded by a thin layer of mesothelial lining expressing the Wilms tumor suppressor WT1 (Bandiera et al. 2013). To determine whether *Rspo1* and *Rspo3* are expressed in the same cell population, we performed cell-sorting experiments on dissociated adult adrenals from *Wt1-GFP* animals (Hosen et al. 2007). Quantitative PCR (qPCR) analysis revealed the majority of *Rspo1* expression to be within the $WT1^+$ population (Supplemental Fig. S1D), which is consistent with previously published data showing a direct regulation of this gene by this

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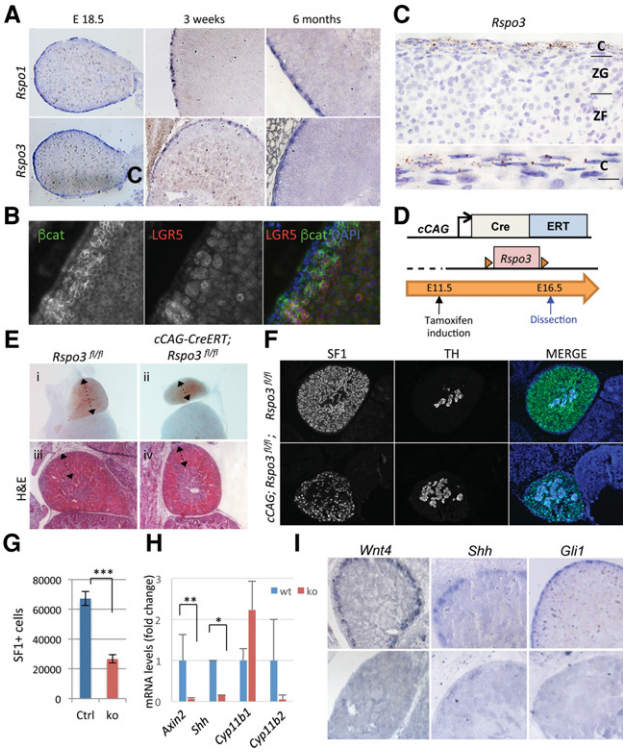

Figure 1. *Rspo3* is expressed in the adrenal capsule and is required for adrenal growth and the onset of zonation. (*A*) In situ hybridization (ISH) analysis reveals *Rspo1* and *Rspo3* in the capsule surrounding the adrenal cortex. (*B*) Immunostaining showing the LGR5 receptor to be enriched in ZG cells that also show strong staining for β-catenin. (*C*) RNA-Scope analysis on adult adrenals. (*D*) Schematic outline of the experimental strategy used. *Rspo3* was deleted by injecting tamoxifen into pregnant mothers at E11.5 and collecting embryos at E16.5. (*E*) Macroscopic analysis (panels *i*,*ii*) and hematoxylin and eosin (H&E) staining (panels *iii*,*iv*) demonstrate a dramatic reduction of adrenal size in the absence of *Rspo3* expression. (CAG) *cCAG-CreERT*. (*F*) Immunofluorescent analysis shows a reduction of adrenal cortex (SF1-positive cells; green) but not medullary (TH; light blue) cells. (*G*) Quantification of SF1-positive cells confirms dramatic loss of the steroidogenic compartment. $n_{wt} = 4$; $n_{ko} = 4$; $P = 8 \times 10^{-4}$, Student's *t*-test. (*H*) Quantitative PCR (qPCR) analysis of RNA extracted from E16.5 adrenals. $n_{wt} = 3$; $n_{ko} = 4$; *Axin2*, $P = 9 \times 10^{-3}$; *Shh*, $P = 0.02$. (*I*) ISH analysis shows complete loss of *Wnt4*, *Shh*, and *Gli1* expression.

transcription factor (Motamedi et al. 2014). In contrast, *Rspo3* appeared to be in a distinct cell population that does not express high levels of WT1. RNA-Scope analysis revealed *Rspo3* to be expressed throughout the capsule (Fig. 1C), reminiscent of the expression pattern of *Gli1* (King et al. 2009) and NR2F2/COUPTFII (Wood et al. 2013). To test whether *Rspo3*-expressing cells may be identical to the *Gli1*-positive population, we used a *Gli1-CreERT2* line in combination with a conditional allele for *Rspo3* (Rocha et al. 2015). Deletion was induced at E14.5 by tamoxifen induction, and samples were collected at E16.5 (Supplemental Fig. S1E). Strikingly, RNA expression analysis showed an almost 70% reduction after deletion, indicating that *Gli1*- and *Rspo3*-expressing cells are largely identical (Supplemental Fig. S1F).

We next used genetic analysis to address whether *R-spondins* may play a direct role in adrenal formation and homeostasis. Deletion of *Rspo1* was compatible with survival (Chassot et al. 2008; Tomizuka et al. 2008) and had no significant effect on adrenal development, zonation, or tissue maintenance (Supplemental Fig. S2). *Rspo3*-deficient embryos die early in development due to defects in placenta formation (Kazanskaya et al. 2008). We therefore resorted to a tamoxifen-inducible approach (Fig. 1D) combining the $Rspo3^{flox}$ allele (Rocha et al. 2015) with the ubiquitously expressed *cCAG-CreERT* driver strain (Hayashi and McMahon 2002). Induced deletion of *Rspo3* at E11.5 resulted in smaller adrenals and thinning of the adrenal cortex at E16.5 (Fig. 1E). Indeed, quantitation of SF1-positive cells revealed a 2.5-fold decrease of the adrenal steroidogenic compartment in the absence of *Rspo3* (Fig. 1F,G).

The dramatic reduction of $SF1^+$ cells in *Rspo3* knockout animals indicated an important function for this gene in the formation of the adult adrenal cortex. Previous studies have demonstrated a key role for SHH signaling in the recruitment of capsular $Gli1^+$ cells to the steroidogenic lineage (Ching and Vilain 2009; King et al. 2009; Huang et al. 2010), and *Shh* has been suggested to be regulated by canonical β-catenin signaling in the adrenal system (Drelon et al. 2015) and several other developmental systems (Iwatsuki et al. 2007; Ahn et al. 2010). To test whether this pathway is affected in *Rspo3* mutants, we carried out qPCR, immunohistochemistry, and in situ hybridization (ISH) analysis on E16.5 tissues. Deletion resulted in a near-complete loss of the β-catenin target *Axin2* (Lustig et al. 2002), thus indicating a requirement of Rspo3 for canonical signaling (Fig. 1H). *Shh* was prominently expressed in steroidogenic cells juxtaposing the capsule of control animals but became almost undetectable in *Rspo3* mutant animals (Fig. 1H,I). Consistent with the loss of SHH signaling, expression of its direct target, *Gli1*, also disappeared from capsular cells after *Rspo3* deletion (Fig. 1I). *Wnt4*, a gene that has been shown to be directly regulated by β-catenin in the kidney (Park et al. 2012), was no longer expressed in the mutant cortex (Fig. 1I).

Taken together, these data suggest a double-paracrine mechanism in adrenal cortex development, in which RSPO3 is released from the capsule to induce β-catenin signaling within juxtaposed steroidogenic cells, leading to the activation of *Shh*. In turn, SHH signals back to capsular cells, where it activates *Gli1* and other downstream targets to recruit cells to the steroidogenic lineage. Loss of *Rspo3* signaling leads to lack of cell recruitment and, as a consequence, a severely reduced adrenal cortex.

Zonation of the adrenal cortex into ZG and ZF commences at around E16.5 in mice and has been suggested to depend on β-catenin signaling. Ubiquitous deletion of *Rspo3* at E11.5 results in heart failure (F Da Silva, AS Rocha, J Ryler, C Basboga, H Morrison, KD Wagner, and A Schedl, in prep.), thus prohibiting the analysis of later time points. We therefore resorted to the capsular-expressed *Gli1-CreERT2* strain and induced deletion at E13.5, which resulted in a lack of *Rspo3* expression at E18.5 (Fig. 2A,B). Immunofluorescent analysis revealed loss of the ZG-specific marker DAB2 (Fig. 2C). In contrast, expression of the ZF-specific markers AKR1B7 and CYP11B1 persisted and now extended throughout the remaining cortex up to the capsule. BrdU labeling experiments confirmed an almost complete loss of proliferation in the adrenal cortex, whereas dividing cells persisted in other tissues (Supplemental Fig. S3). NR2F2 expression persisted in the capsule (Fig. 2C), although the number of cells also appeared to be somewhat reduced.

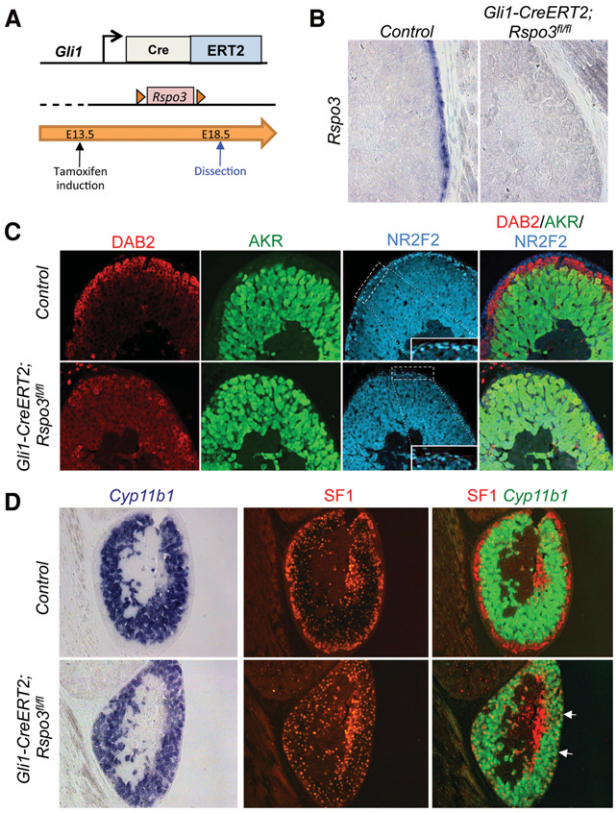

Figure 2. Deletion of RSPO3 interferes with the development of adrenal zonation. (*A*) Experimental setup using *Rspo3fl/fl*; *Gli1-CreERT2* animals. (*B*) ISH analysis at E18.5 showing the complete loss of *Rspo3* expression in mutant animals. (*C*) Immunofluorescent analysis demonstrates loss of the ZG marker DAB2 and an expansion of AKR1B7 up to the capsule. NR2F2-positive capsular cells are maintained in mutant animals. (*D*) *Cyp11b1* ISH, SF1 antibody staining, and a digital overlay of the two data sets. The ZF marker *Cyp11b1* also extends to the capsule in mutant animals (white arrows).

To test whether continuous RSPO3 signaling was required to maintain adrenal homeostasis, we induced Cre recombination (*cCAG-CreERT* driven) in adult mice (12 wk) and sacrificed animals at various time points after induction (Fig. 3A). ISH confirmed near-complete loss of *Rspo3* expression in the adrenal capsule (Fig. 3B; Supplemental Fig. S4B). Adrenal weight in *Rspo3* mutants progressively decreased over time and dropped to 50% of that in control animals 6 wk after induction (Fig. 3C). Histological analyses confirmed a severely reduced adrenal cortex, while the medulla appeared unaffected (Fig. 3D). High-power views revealed loss of the typical ZG organization in arches. Instead, the remaining cortical cells showed the columnar arrangement reminiscent of the ZF. To test whether mutant adrenals showed reduced proliferation, we performed long-term BrdU tracing experiments. In control animals, a large number of SF1/BrdU double-positive cells were detected, indicating that they underwent cell division during the labelling period (Fig. 3E). In contrast, BrdU-labeled cells were dramatically reduced after *Rspo3* deletion, and SF1/BrdU double-positive cells were almost absent.

To confirm the loss of ZG differentiation, we next analyzed a range of molecular markers using ISH and immunostaining. *Wnt4*, *Shh*, *Gli1* (Fig. 4A), DAB2, and CYP11B2 (Fig. 4B,C) were dramatically reduced in mutant mice. Similar to development, the ZF marker AKR1B7 now extended up to the capsule (Fig. 4B). qPCR assays confirmed these findings with no alteration in ZF markers and an almost complete loss of ZG marker genes (Fig. 4B).

To exclude secondary effects that might have been caused by systemic deletion of *Rspo3*, we next performed tissue-specific deletion experiments using the *Gli1-CreERT2* strain (Supplemental Fig. S4A). qPCR analysis revealed a reduction of *Rspo3* expression to ~30% of wild-type levels, which is somewhat lower than what we observed using the ubiquitously expressed *cCAG-CreERT* line (Supplemental Fig. S4B). The less efficient deletion is likely to be due to lower levels of expression from the *Gli1* promoter. *Axin2* expression levels closely followed those of *Rspo3* (Supplemental Fig. S4A). Importantly, *Gli1-CreERT2*-specific deletion also caused a reduction of the adrenal cortex and the loss of ZG markers (Supplemental Fig. S4C,D), indicating that, indeed, zonation depends on capsular expression of *Rspo3*.

The striking loss of ZG markers could be caused by either apoptosis of ZG cells, lack of cell renewal, or a continuous requirement for RSPO3 signaling to specify ZG cells. To distinguish between these alternatives, we analyzed

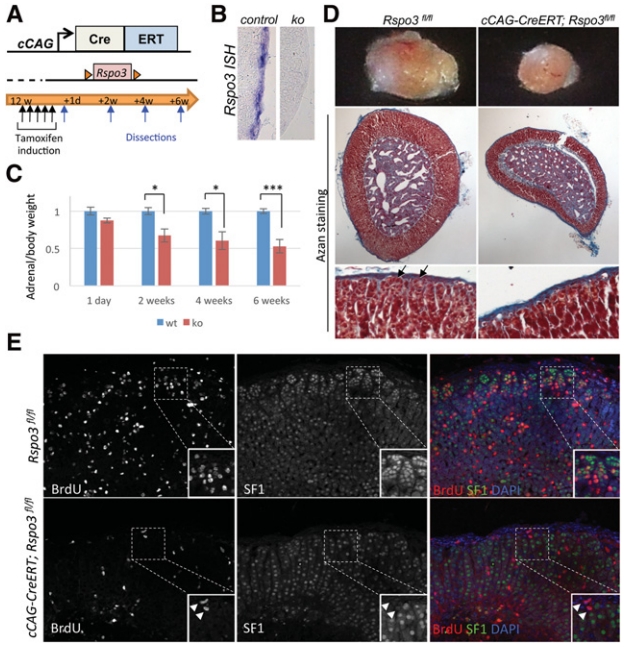

Figure 3. Continuous RSPO3 signaling is required for adrenal homeostasis. (*A*) Experimental design. Twelve-week-old mice were induced and analyzed at various time points. (*B*) Analysis at 4 wk demonstrates a complete loss of capsular *Rspo3* expression upon tamoxifen induction. (*C*) Loss of *Rspo3* leads to progressive loss of adrenal weight. 1 d, $n_{wt} = 6$, $n_{ko} = 6$; 2 wk, $n_{wt} = 6$, $n_{ko} = 6$; 4 wk, $n_{wt} = 4$, $n_{ko} = 5$; 6 wk, $n_{wt} = 12$, $n_{ko} = 15$. 2 wk, $P = 1.5 \times 10^{-3}$; 4 wk, $P = 2.6 \times 10^{-3}$; 6 wk, $P = 3.0 \times 10^{-8}$, Student's *t*-test. (*D*) Macroscopic and histological examination (Azan staining) 6 wk after *Rspo3* deletion reveals smaller adrenal glands and the dramatic thinning and loss of the ZG typical arrangement of cells in arches (black arrows). (*E*) BrdU analysis (40 d) reveals a dramatic loss of proliferation after *Rspo3* deletion. The only labeled cells in *Rspo3* knockout animals (white arrowheads) are nonsteroidogenic, as indicated by the absence of SF1.

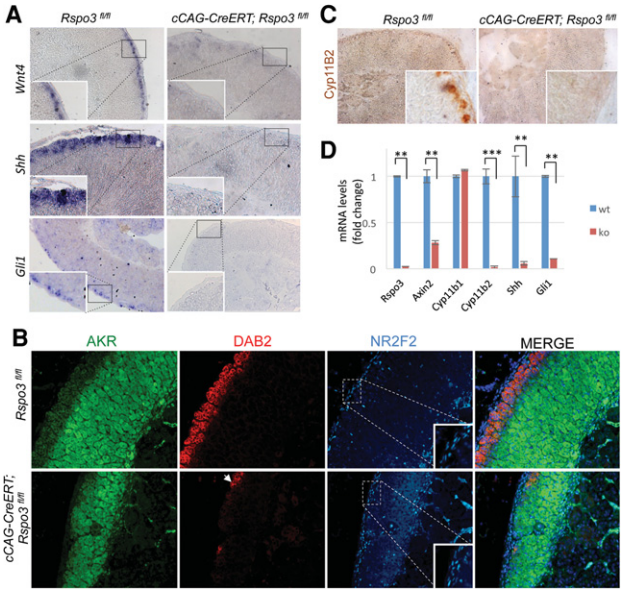

Figure 4. Loss of *Rspo3* abolishes adrenal zonation. ISH (*A*), immunofluorescent (*B*), and immunohistochemical (*C*) analyses reveal loss of all ZG-specific markers analyzed. Note the persistence of pockets of DAB2 expression (white arrowhead in *B*) that are likely associated with incomplete deletion of *Rspo3*. (*B*) NR2F2 capsular cells appear to be largely unaffected. (*D*) qPCR analysis confirms the dramatic reduction of expression of the β-catenin target *Axin2* and a near loss of ZG markers *Cyp11B2* and *Shh* as well as the capsular marker *Gli1*. $n_{wt} = 5$; $n_{ko} = 5$. *Rspo3*, $P = 8 \times 10^{-3}$; *Axin2*, $P = 1 \times 10^{-3}$; *Cyp11B2*, $P = 3 \times 10^{-5}$; *Shh*, $P = 2 \times 10^{-3}$; *Gli1*, $P = 5 \times 10^{-3}$, Student's *t*-test.

samples 1 d after completion of the tamoxifen deletion protocol (Fig. 5), in which lack of cell renewal can be excluded. Knockout adrenals at this early time point showed only a very mild reduction in size that did not reach statistical significance (Fig. 3C). Caspase3 analysis identified a number of apoptotic cells close to the medulla (Fig. 5B), which is consistent with the centripetal model of cell renewal (Freedman et al. 2013). Deletion of *Rspo3* did not increase apoptosis, thus excluding cell death as a reason for the loss of ZG markers. Strikingly, molecular analysis confirmed a near-complete loss of all ZG markers analyzed (*Shh*, *Wnt4*, *DAB2*, and *Cyp11b2*) even at this early time point after *Rspo3* deletion (Fig. 5C). The absence of apoptosis in steroidogenic cells after *Rspo3* deletion and the expansion of ZF markers up to the capsule at 4 wk after deletion suggest that ZG cells convert to ZF cells, a process that is also occurring under normal conditions when steroidogenic cells move centripetally, thus losing signals derived from the adrenal capsule (Freedman et al. 2013).

Wnt4 has been demonstrated previously to be required for the activation of *Cyp11b2* expression (Heikkila et al. 2002), and we showed recently that this dependence also persists in adult mice (C Drelon, A Berthon, I Sahut-Barnola, M Mathieu, T Dumontet, S Rodriguez, M Batisse-Lignier, H Tabbal, I Tauveron, AM Lefrançois-Martinez, et al., in prep.). To test whether deletion of *Wnt4* may affect RSPO3-controlled signaling pathways and cell renewal, we analyzed *Wnt4; Sf1-Cre* knockout mice (Supplemental Fig. S5A) that showed efficient depletion of *Wnt4* in adrenal glands (Supplemental Fig. S5C). Surprisingly, mice survived into adulthood (>12 mo) with no overt defects detectable. Adrenals in *Wnt4* knockout mice were only slightly smaller (Supplemental Fig. S5B), demonstrating that this gene does not play a major role in cell renewal. Molecular analysis revealed a significant reduction of the ZG markers *Axin2* and *At1b* but only a mild reduction of *Rspo3*, *Shh*, and *Gli1* expression that did not reach statistical significance (Supplemental Fig. S5C,D). Thus, *Wnt4* appears to act as a local relay signal that reinforces β-catenin activity in the ZG but does not have a major influence on organ renewal.

A recent study suggested RSPO3 to bind to LGR5 and induce noncanonical signaling in adrenal cortical cells. This signaling appeared to inhibit aldosterone production and cell renewal and induce apoptosis (Shaikh et al. 2015). These findings are in stark contrast to the data presented in this study. We presume the discrepancy to be caused by the in vitro setting of the previously reported study, which does not take into account the complex interactions of cells in an in vivo environment. Our data clearly demonstrate a canonical role for *Rspo3* signaling within the adrenal cortex, where it induces well-established direct downstream targets of β-catenin (*Axin2* and *Dab2*) and maintains organ size in the adult organism. This role is consistent with the generally accepted function of RSPO/LGR5 signaling in controlling the activation of the stem cell pool, and we can now add the adrenal cortex as an additional organ in which this pathway is used.

Even more surprising than the involvement of RSPO3 in controlling organ renewal is its absolute requirement for maintaining adrenal zonation. The rapid loss of zonation markers after *Rspo3* deletion in the adult demonstrates that it acts as an instructive signal to direct

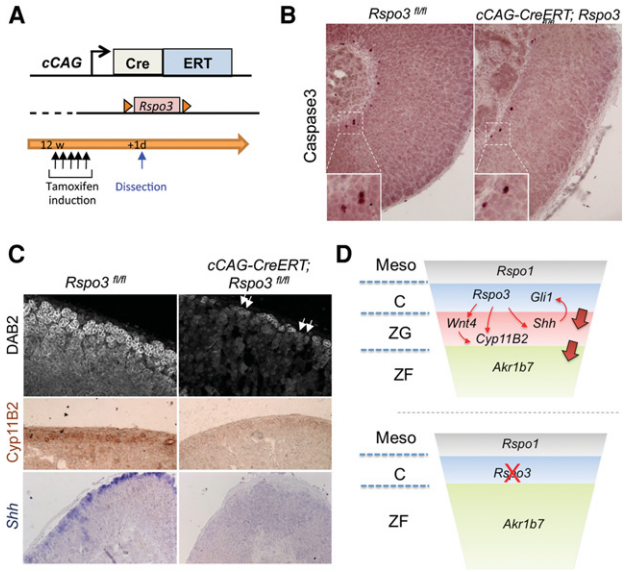

Figure 5. *Rspo3* deletion leads to a rapid loss of ZG identity. (*A*) Experimental design. (*B*) Caspase staining revealed some apoptotic cells close to the medulla, whereas the ZG was devoid even after deletion of Rspo3. (*C*) ISH and immunostaining demonstrated a rapid loss of ZG marker gene expression. Note the complete loss of DAB2 in a proportion of subcapsular cells (white arrow) upon *Rspo3* deletion. (*D*) Model describing the central role of *Rspo3* in establishing adrenal zonation and ensuring cell renewal. Large red arrows symbolize the centripetal movement of cells during cell renewal. (Meso) Mesothelial lining; (C) capsule.

steroidogenic cells toward the ZG phenotype. The adrenal capsule is the ideal location to provide this stimulus. Since R-spondins appear to be tightly bound to the extracellular matrix (Kim et al. 2005), diffusion is limited, and only several cell layers are instructed to express ZG markers. Activation of *Wnt4* within the ZG cells is likely to serve as a local reinforcement signal, as loss of *Wnt4* also leads to a reduction of ZG markers (Supplemental Fig. S5; Heikkila et al. 2002).

Apart from the adrenal cortex, hepatocytes also display metabolic zonation along the liver lobule that is set up by a gradient of canonical β-catenin signaling (Benhamouche et al. 2006). We showed recently that, in a fashion similar to the adrenal, liver zonation strictly depends on RSPO3 that is released from endothelial cells located in the central vein (Rocha et al. 2015). Thus, our evidence suggests that the liver and adrenal cortex have adopted the same signaling system to establish organ-specific zonation despite the fact that both organs are derived from distinct embryological origins.

In conclusion, our data establish a genetic network in which *Rspo3* induces *Wnt4* and *Shh* expression within steroidogenic cells that are in close contact with the capsule (Fig. 5D). *Shh* in turn signals back to recruit capsular cells to form the adrenal cortex, at least during development. The fact that *Shh* knockout mice show normal adrenal zonation (Huang et al. 2010) while *Rspo3* deletion affects ZG identity indicates that *Rspo3* must also act through additional, *Shh*-independent pathways. The dramatic thinning of the entire steroidogenic compartment further supports the model of a ZG-driven adrenal cortex renewal, a concept that has been put forward by Freedman et al. (2013). Thus, our study identifies the adrenal capsule as a crucial signaling center that, through the production of RSPO3, not only ensures organ renewal but also provides an instructive signal that is continuously required for proper zonation.

Materials and methods

Mouse work

All animal work was conducted according to national and international guidelines and was approved by the local ethics committee (PEA-NCE/2013-88). The *Rspo1* (Chassot et al. 2008), *Rspo3^{flox}* (Rocha et al. 2015), *Wt1-GFP* (Hosen et al. 2007), *Gli1-CreERT2* (Ahn and Joyner 2004), *cCAG-CreERT* (Hayashi and McMahon 2002), *Sf1-Cre* (Bingham et al. 2006), and *Wnt4^{flox}* (Shan et al. 2009) lines have been described previously. Cre activation during development was obtained by a single tamoxifen administration (force feeding) of pregnant females carrying either E11.5, E13.5, or E14.5 embryos. Cre activation in adult mice was obtained by intraperitoneal injection of 12-wk-old mice with 1 mg of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) per 20 g of body weight five times a week for 1 wk. With the exception of embryonic analysis, only male mice were used for the described experiments.

For proliferation assays, 1 mg/mL BrdU was added to drinking water, the solution was changed every 2 d, and animals were sacrificed at the end of 4 wk of treatment.

Cell sorting

Adrenals from adult WT1GFP or control animals were collected and incubated for 30 min in 1 mg/mL collagenase at 37°C, and the adrenal capsule was separated from the rest of the adrenal using a binocular microscope. After a further 30 min of incubation in trypsin at 37°C with occasional pipetting up and down, the digestion was stopped by adding 100 µL of FBS, and the cell suspension was filtered (40-µm filters), centrifuged (2000 rpm for 3 min), and resuspended in 200 µL of PBS-EDTA-serum solution. Cells were analyzed with a FACS Aria II (BD).

Immunofluorescence and histological analysis

For immunofluorescence experiments, tissues were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and paraffin-embedded. Seven-micrometer-thick sections were rehydrated, boiled in a pressure cooker for 2 min with antigen unmasking solution (Vector Laboratories), and blocked in a PBS solution containing 10% normal donkey serum (Jackson Immunoresearch) and 3% BSA. All primary antibodies were applied overnight at 4°C at the concentrations listed in the antibody table in the Supplemental Material. Secondary antibodies were diluted 1:200 and applied at room temperature for 1 h. β-Catenin was detected using a biotinylated secondary antibody followed by streptavidin-Cy3 detection (Sigma-Aldrich).

For histological analysis, adrenal glands from embryos or adult mice were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and embedded in paraffin. Seven-micrometer-thick sections were then stained with hematoxylin and eosin according to standard procedures.

For CYP11B2 and caspase3 staining, sections were unmasked, incubated for 30 min in H_2O_2 (30%), and blocked for 1 h in 5% PBS/BSA. Primary antibody was left overnight at 4°C in 0.1% PBS/BSA, and polymer-Hrp rabbit (Cell Signaling, 8114) was used as a secondary and incubated for 30 min at room temperature. Revelation was performed with a Novared kit (Vector Laboratories) for 4 min.

Azan staining

Histological sections at 5 µm were stained using the Azan trichrome kit (Diapath) according to the manufacturer's instructions.

RNA ISH

Tissues were fixed overnight in 4% paraformaldehyde, progressively dehydrated, and paraffin-embedded. The *Gli1* in situ probe was kindly provided by M. Studer (Institute of Biology Valrose, Université de Nice-Sophia Antipolis). Seven-micrometer-thick sections were hybridized with *Rspo1*, *Rspo3*, *Wnt4*, and *Shh* as well as *Gli1* probes according to previously described protocols (Comai et al. 2010). RNA-Scope analysis for *Rspo3* was performed on adult sections according to the manufacturer's instructions.

Real-time PCR analysis

RNA was extracted from E16.5 or adult adrenal glands using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) in combination with random primers. The cDNA obtained was then used as template for qPCR analysis using the SYBR Green master kit (Roche) and a LightCycler 1.5 (Roche). The expression levels were normalized for *Hprt*. Primers (see the primer table in the Supplemental Material) were designed on the Universal ProbeLibrary Web site (Roche).

Statistical analysis

Statistical significance was determined using two-tailed Student's *t*-test. Error bars in all figures represent the standard error of mean, and *P*-values are indicated by asterisks as follows: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

For the developmental analysis (Fig. 1G), SF1-positive cells were counted on every second section of four control and four knockout adrenals using Volocity 6.3 software. Statistical analysis was performed with the total of nuclei obtained for each adrenal using a two-tailed Student's *t*-test.

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References

Ahn S, Joyner AL. 2004. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell* **118:** 505–516.

Ahn Y, Sanderson BW, Klein OD, Krumlauf R. 2010. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* **137:** 3221–3231.

Bandiera R, Vidal VP, Motamedi FJ, Clarkson M, Sahut-Barnola I, von Gise A, Pu WT, Hohenstein P, Martinez A, Schedl A. 2013. WT1 maintains adrenal-gonadal primordium identity and marks a population of AGP-like progenitors within the adrenal gland. *Dev Cell* **27:** 5–18.

Benhamouche S, Decaens T, Godard C, Chambrey R, Rickman DS, Moinard C, Vasseur-Cognet M, Kuo CJ, Kahn A, Perret C, et al. 2006. Apc tumor suppressor gene is the 'zonation-keeper' of mouse liver. *Dev Cell* **10:** 759–770.

Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Joussineau C, Damon-Soubeyrand C, Louiset E, Taketo MM, Tissier F, Bertherat J, Lefrancois-Martinez AM, et al. 2010. Constitutive β-catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. *Hum Mol Genet* **19:** 1561–1576.

Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, Samson-Couterie B, Zennaro MC, Plouin PF, Skah S, et al. 2014. WNT/β-catenin signalling is activated in aldosterone-producing adenomas and controls aldosterone production. *Hum Mol Genet* **23:** 889–905.

Bingham NC, Verma-Kurvari S, Parada LF, Parker KL. 2006. Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* **44:** 419–424.

Chassot AA, Ranc F, Gregoire EP, Roepers-Gajadien HL, Taketo MM, Camerino G, de Rooij DG, Schedl A, Chaboissier MC. 2008. Activation of β-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Hum Mol Genet* **17:** 1264–1277.

Ching S, Vilain E. 2009. Targeted disruption of Sonic Hedgehog in the mouse adrenal leads to adrenocortical hypoplasia. *Genesis* **47:** 628–637.

Comai G, Boutet A, Neirijnck Y, Schedl A. 2010. Expression patterns of the Wtx/Amer gene family during mouse embryonic development. *Dev Dyn* **239:** 1867–1878.

de Lau W, Peng WC, Gros P, Clevers H. 2014. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. *Genes Dev* **28:** 305–316.

Drelon C, Berthon A, Mathieu M, Martinez A, Val P. 2015. Adrenal cortex tissue homeostasis and zonation: a WNT perspective. *Mol Cell Endocrinol* **408:** 156–164.

Freedman BD, Kempna PB, Carlone DL, Shah MS, Guagliardo NA, Barrett PQ, Gomez-Sanchez CE, Majzoub JA, Breault DT. 2013. Adrenocortical zonation results from lineage conversion of differentiated zona glomerulosa cells. *Dev Cell* **26:** 666–673.

Hayashi S, McMahon AP. 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* **244:** 305–318.

Heikkila M, Peltoketo H, Leppaluoto J, Ilves M, Vuolteenaho O, Vainio S. 2002. Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology* **143:** 4358–4365.

Hosen N, Shirakata T, Nishida S, Yanagihara M, Tsuboi A, Kawakami M, Oji Y, Oka Y, Okabe M, Tan B, et al. 2007. The Wilms' tumor gene WT1-GFP knock-in mouse reveals the dynamic regulation of WT1 expression in normal and leukemic hematopoiesis. *Leukemia* **21:** 1783–1791.

Huang CC, Miyagawa S, Matsumaru D, Parker KL, Yao HH. 2010. Progenitor cell expansion and organ size of mouse adrenal is regulated by sonic hedgehog. *Endocrinology* **151:** 1119–1128.

Iwatsuki K, Liu HX, Gronder A, Singer MA, Lane TF, Grosschedl R, Mistretta CM, Margolskee RF. 2007. Wnt signaling interacts with Shh to regulate taste papilla development. *Proc Natl Acad Sci* **104:** 2253–2258.

Kazanskaya O, Ohkawara B, Heroult M, Wu W, Maltry N, Augustin HG, Niehrs C. 2008. The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development* **135:** 3655–3664.

Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, et al. 2005. Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* **309:** 1256–1259.

Kim AC, Reuter AL, Zubair M, Else T, Serecky K, Bingham NC, Lavery GG, Parker KL, Hammer GD. 2008. Targeted disruption of β-catenin in Sf1-expressing cells impairs development and maintenance of the adrenal cortex. *Development* **135:** 2593–2602.

King P, Paul A, Laufer E. 2009. Shh signaling regulates adrenocortical development and identifies progenitors of steroidogenic lineages. *Proc Natl Acad Sci* **106:** 21185–21190.

Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, et al. 2002. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* **22:** 1184–1193.

Motamedi FJ, Badro DA, Clarkson M, Lecca MR, Bradford ST, Buske FA, Saar K, Hubner N, Brandli AW, Schedl A. 2014. WT1 controls antagonistic FGF and BMP–pSMAD pathways in early renal progenitors. *Nat Commun* **5:** 4444.

Park JS, Ma W, O'Brien LL, Chung E, Guo JJ, Cheng JG, Valerius MT, McMahon JA, Wong WH, McMahon AP. 2012. Six2 and Wnt regulate self-renewal and commitment of nephron progenitors through shared gene regulatory networks. *Dev Cell* **23:** 637–651.

Rocha AS, Vidal V, Mertz M, Kendall TJ, Charlet A, Okamoto H, Schedl A. 2015. The angiocrine factor Rspondin3 is a key determinant of liver zonation. *Cell Rep* **13:** 1757–1764.

Shaikh LH, Zhou J, Teo AE, Garg S, Neogi SG, Figg N, Yeo GS, Yu H, Maguire JJ, Zhao W, et al. 2015. LGR5 activates noncanonical Wnt signaling and inhibits aldosterone production in the human adrenal. *J Clin Endocrinol Metab* **100:** E836–E844.

Shan J, Jokela T, Peltoketo H, Vainio S. 2009. Generation of an allele to inactivate Wnt4 gene function conditionally in the mouse. *Genesis* **47:** 782–788.

Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, Kojima A, Yoshitome A, Yamawaki K, Amagai M, Inoue A, et al. 2008. R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum Mol Genet* **17:** 1278–1291.

Walczak EM, Kuick R, Finco I, Bohin N, Hrycaj SM, Wellik DM, Hammer GD. 2014. Wnt signaling inhibits adrenal steroidogenesis by cell-autonomous and non-cell-autonomous mechanisms. *Mol Endocrinol* **28:** 1471–1486.

Wood MA, Acharya A, Finco I, Swonger JM, Elston MJ, Tallquist MD, Hammer GD. 2013. Fetal adrenal capsular cells serve as progenitor cells for steroidogenic and stromal adrenocortical cell lineages in *M. musculus*. *Development* **140:** 4522–4532.

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